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# ES Cell-Mediated Conditional Transgenesis

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## 1. Introduction

The use of mouse embryonic stem (ES) cells to alter the mouse genome has become a routine method to study gene function alongside classical transgenesis. Gene targeting by homologous recombination is the most widely used method of ES cell-mediated genome alterations. It allows the introduction of specific mutations of a gene of interest into the mouse germ line. ES cells can be used to mediate random insertional transgenesis as well, which is perhaps an alternative to the pronuclear DNA injection method. However, the combination of recently developed different site-specific recombinase systems combined with ES cell technology makes it possible to create more sophisticated conditional transgenic and conditional gene knock-out mouse models.

The site-specific recombinases catalyze the recombination between two consensus DNA sequences. If these sites are properly designed into a transgene or a gene-targeting vector, the site-specific recombination event can trigger the expression of a transgene. It can also create a loss-of-function allele of the targeted gene in a specific cell lineage and/or in a specific time of development, conditional to the expression of the recombinase. Cre recombinase of the bacteriophage P1 is the most widely used in mouse genetics (see detailed review in *ref. 1*). This prokaryotic enzyme was first shown to work in the mouse by Lakso et al. (2). Cre protein recombinates DNA between two 34-bp long *loxP* recognition sites. If these sites are placed in the same DNA strand, in the same orientation, the recombination results in the excision of the intervening sequence, leaving a single *loxP* site behind.

The second most popular is Flp recombinase from yeast. Concerning its mechanism of action, it is similar to *Cre/loxP*, but so far appears to be less efficient. However, recently developed enhanced Flp (Flpe) might change this situation (3,4). The 34-bp consensus recombination site of Flp is called FRT.

The third potential enzyme is still in the earliest stage of its development for use in mammalian genome alterations, but certainly holds promise that it could become an additional recombinase system. This is an integrase from *Streptomyces* phage  $\phi$ C31, which was recently demonstrated to function in human cells (5). It carries out an efficient site-specific unidirectional recombination between attP and attB sites. It presently seems as if this integrase can be used to delete sequences flanked by attP

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and attB sites. It is also possible that this could become the system of choice for recombinase-mediated site-specific insertion into the genome.

After mediating an insertion into the genome, both Cre and FLP, create two functional *loxP* or FRT sites, respectively, that flank the inserted sequence. Obviously, these sites could be the target of a second recombination, which will excise the inserted sequence. In contrast,  $\phi$ C31-mediated recombination between the attP and attB sites does not recreate either site, and therefore, no further recombination that could remove the inserted sequence will occur.

In the ideal but not at all unrealistic picture for the near future, if two recombinase systems are working in addition to the *Cre/loxP* system, there will be more freedom for the Cre system to be used as a postintegration switch. There is already a large collection of transgenic mouse lines expressing Cre recombinase at a high level and specificity, and there are more to come. The database presenting a collection of Cre transgenic lines created through the effort of many researchers can be found at (<http://www.mshri.on.ca/nagy/Cre.html>). Such transgenic lines expressing Cre recombinase driven by lineage-tissue-specific promoters can be derived by classical transgenesis as well as by the relatively new approach of gene-targeted knock-in of the Cre recombinase into an endogenous gene for a sometimes more reliable expression.

To be able to control the time of the switch, independently of the endogenous regulatory elements and lineage specificity, recombinase systems are combined with inducible gene expression systems. The recombinase gene can be placed under the control of an inducible promoter (ubiquitous or lineage-specific) or constructed as an inducible fusion protein.

Tamoxifen (6) and RU-486 (7) inducible systems use the nuclear localization capability of estrogen or a progesterone receptor ligand-binding domain in the presence of the ligand. The Cre recombinase is fused to a mutant ligand-binding domain, which has lost its ability to bind endogenous estrogen or progesterone, but still binds tamoxifen (an estrogen antagonist) or RU-486 (a synthetic steroid), respectively. In the presence of the synthetic ligand, the Cre fusion protein translocates into the nucleus and executes its function. So far, only partial tamoxifen-inducible Cre-mediated excision has been obtained (8).

The tetracycline-inducible gene expression system uses the DNA-binding domain of the bacterial tet-repressor protein and a strong transcriptional activator domain (VP16 from the herpes virus), which are fused together. Such a heterologous protein can bind to the tetracycline operator element and activate transcription depending on the presence of tetracycline (9). The combination of *Cre/loxP* and the doxycycline (Dox) system has proven promising (10). However, problems with mosaic expression, toxic effects of the transactivator, and a high background recombination level have been encountered. The fine tuning of that system has required quite a bit of time. Recently, however, ubiquitous Cre recombination was achieved upon per oral administration of antibiotic using a Dox inducible single-construct Cre transgene (11). The authors placed both the Cre recombinase and the reverse tetracycline-dependent transactivator (rtTA) under the control of the same bidirectional Dox responsive promoter. In this arrangement, the transcription is auto-inducible depending on the availability of Dox and minimal amounts of rtTA. Both rtTA toxicity and background Cre recombination were found minimal in the absence of Dox.

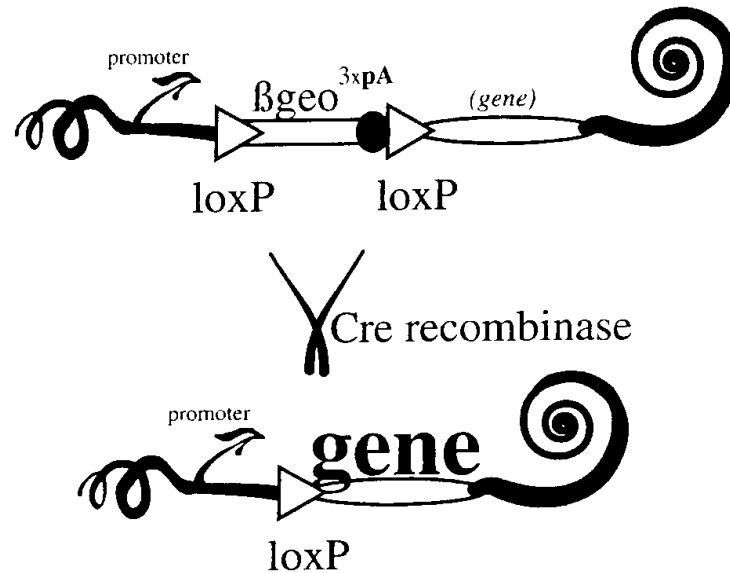


Fig. 1. Prototype construct for conditional transgenic expression of a gene of interest mediated by a Cre recombinase.

The efficiency and specificity of any recombinase system needs to be tested at the cellular level using a transgenic reporter mouse line (i.e., a transgenic line expressing a reporter gene in response to Cre-mediated recombination). For that, a single-copy transgene should have a ubiquitous promoter, followed by a *loxP*-flanked transcriptional stop region, and then the coding region of a reporter gene. The reporter initiates expression under the control of the promoter only after Cre excision removes the stop region. Such a conditional *lacZ* reporter construct was introduced into the ROSA26 gene-trap integration site (12). The other recently developed system (Z/AP) uses two reporters: cells express *lacZ* before Cre excision and heat-resistant human alkaline phosphatase after excision. This was achieved by random insertion of the transgene and single-copy integration by ES cell-mediated transgenesis (13).

ES cell-mediated as opposed to classical transgenesis has an advantage of a higher frequency of single-copy integration. It is an important issue, since multiple-copy integration can create more than two *loxP* sites and a potentially unpredictable outcome of the Cre excision and chromosome instability (14).

Conditional transgenesis is based on a similar strategy to that behind the Cre reporter lines: the promoter and the coding region for the gene of interest are separated by a *loxP*-flanked stop region that does not allow any transcription or translation of the gene of interest. The gene is expressed when this region is removed by Cre-mediated excision (Fig. 1). A ubiquitously expressed conditional transgenic line with single-copy integration is produced using ES cells. The transgene is activated when crossed with various Cre transgenic lines of different lineage specificity. Cre-mediated recombination, and therefore transgene activation, can also be inducible as discussed above.

Here, we describe our example applied in the design of the Z/AP reporter, which can be followed for any conditional transgenesis (Fig. 1). The  $\beta$ geo-*lacZ*/neo<sup>R</sup> fusion

coding sequence (15) is placed upstream of a triple repeat of the simian virus 40 (SV40) polyadenylation signal (3xpA). The  $\beta$ geo/3xpA is inserted between two *loxP* sites that are placed in a pCAGG vector (16) containing the cytomegalovirus (CMV) enhancer/chicken  $\beta$ -actin promoter in front of it. This vector is referred as pCALL (13). The coding sequence of the transgene can be inserted downstream of *loxP*-flanked  $\beta$ geo/3xpA to produce an expression vector of interest.

There are several variations of the construct for random integration into ES cells to generate a conditional transgenic mouse line. Alterations of the Z/AP construct can be customized for specific needs. Different positive selectable markers, such as hygromycin B and puromycin, allowing the survival of cells integrating the marker into the genome, can be used instead of neomycin. Among the possible reporter systems, bacterial  $\beta$ -galactosidase ( $\beta$ -gal) and human alkaline phosphatase allow the detection at a single cell level in histological sections. Green fluorescent protein (GFP) cloned from jellyfish, *Aequorea victoria* as well as its variants, yellow and cyan fluorescent proteins, permit the detection of gene expression and protein localization in living cells.

Perhaps the same principles are used for targeted insertion of conditional transgenes into an endogenous gene, but it requires significant target vector building and screening for targeted events. One of the strategies for the creation of a conditional gene knock-out is to place two *loxP* sites around a functionally essential part of the gene of interest, using gene targeting in ES cells (see review in ref. 1). Such minimal modification should leave the gene functional until lineage specific and/or inducible Cre recombinase is applied. This strategy requires a sophisticated gene targeting design, involving the proper insertion of *loxP* sites around the functional part of the gene to create the null allele. The selectable marker needs to be flanked with target sites for its later removal, preferably by a different recombinase system (Flp/FRT) to avoid multiple *loxP* sites. General gene targeting strategies and the use of site-specific recombination are described in great detail in many publications (e.g., ref. 17) and are out of the scope of the current chapter.

Here, we give a brief description of the steps involved in the creation of conditional transgenic animals.

## 2. Materials

### 2.1. ES Cell Tissue Culture

#### 2.1.1. Equipment

1. Tissue culture facility, preferably used only for ES cells, including a laminar flow cabinet, humidified incubator (37°C, 5% CO<sub>2</sub>), inverted phase-contrast microscope with 4 $\times$ , 10 $\times$ , 20–25 $\times$  objectives, stereomicroscope (dissecting) with transmitted light base, table-top centrifuge, water bath (optional), –70°C freezer, liquid nitrogen tanks. Microscope equipped with fluorescence, appropriate filters, and camera (e.g., Leica MZFLIII or universal fluorescence light source from BLS Ltd, Hungary [www.bls-ltd.com], e-mail: bls@euroweb.hu) is necessary if fluorescent proteins are to be used as reporters.
2. Sterile disposable cell culture plasticware (100-, 60-, 35-mm dishes, 6-well, 24-well, 4-well, flat and V-bottom 96-well plates, centrifuge tubes, cryovials). Various sterile disposable or reusable detergent-free glass pipets.
3. Electroporation apparatus (e.g., Bio-Rad Gene Pulser®, cat. no. 165-2106), and capacitance extender (cat. no. 165-21080). 4-cm electrode gap electroporation cuvettes (e.g., Bio-Rad, cat. no. 165-2088).

4. Multichannel pipettor with volume adjustable up to 200  $\mu$ L, sterile disposable reagent reservoirs for multichannel pipettor (e.g., Costar, cat. no. 4870), and sterile pipet tips.
5. Multichannel aspirator system (optional) (e.g., Inotech Biosystems Vacuset®).
6. Isopropanol freezing container (optional) and/or styrofoam box with lid.

### 2.1.2. ES Cells and Feeders

Several ES cell lines allowing efficient germline transmission have been developed (see *ref. 17* for review). To maintain pluripotency, ES cells are cultured on feeder cells: primary embryonic fibroblasts (Emfi) or STO fibroblast cell line; or on gelatinized plates in the presence of leukemia inhibitory factor (LIF). Growth media and culture conditions should be used as suggested for each ES cell line. Here we describe the protocols established and currently used for R1 ES cells (*18*). Mitotically inactivated Emfi are used as feeders only for a long-term culture of R1 ES cells (typically before and after cryopreservation). Otherwise, culture of R1 ES cells for electroporation and during the selection is done on gelatinized plates. Emfi cells can be made from any strain of mice including transgenic mice that express bacterial neomycin or hygromycin genes depending on the choice of selectable markers used for altering the ES cell genome. Neo<sup>R</sup> and Hygro<sup>R</sup> mice are available from Jackson Laboratories (cat. nos. JR2354 and JR2356). Detailed protocols for preparation of Emfi stocks and mitomycin C-treated feeders for ES cell culture are presented in many publications (e.g., *ref. 17*). Our brief protocols are given in **Tables 1** and **2**. Emfi are also commercially available from Specialty Media (cat. no. PMEF).

### 2.1.3. ES Cell Culture Media and Reagents

It is recommended to use only tissue culture grade or, preferably, ES cell-qualified reagents (e.g., from Life Technologies/Gibco or Specialty Media) whenever available. The water quality is a critical factor for optimal culture. It should be from a regularly maintained Milli-Q (Millipore) filtration system, preferably pretreated by deionization. Commercially available ultrapure water (e.g., Gibco or Sigma) can be used as an alternative. If large quantities of media and solutions are necessary, it can be prepared from powder and filter-sterilized. Otherwise, commercially available ES cell-qualified solutions are recommended.

#### 1. Complete ES Medium (ES-DMEM):

- a. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose (high glucose), buffered with 2.2 g/L sodium bicarbonate (Life Technologies, cat. no. 1400-061 [powder], Gibco KO-DMEM, cat. no. 10829; Specialty Media, cat. no. SLM-220B).

Store in the dark at 4°C.

Prior to use, DMEM should be supplemented with the components listed below. If the complete media is stored for longer than 2 wk, it should be supplemented with additional 2 mM L-glutamine from 100X stock, as L-glutamine is unstable. GlutaMAX<sup>TM</sup> media from Gibco (cat. no. 10566) contains L-glutamine in a stabilized form of the dipeptide and can be used without extra supplements of L-glutamine.

- b. 15% ES cell-qualified fetal bovine serum (FBS) (HyClone; Gibco; Specialty Media; Gemini; Wisent, Quebec). Store main stock in the dark at -20°C. Heat-inactivate at 56°C for 30 min (optional) (*see Note 1*).
- c. 0.1 mM Nonessential amino acids (100X stock; Gibco, cat. no. 11140; or Specialty Media, cat. no. TMS-001-C) stored at 4°C.

**Table 1**  
**Preparation of Primary Embryonic Fibroblast Cell (Emfi) Stocks**

Materials	Procedure
1. 15.5-17.5 dpc pregnant mice	1. Aseptically dissect fetuses from 1 to 2 mice in 100-mm Petri dish containing PBS.
2. Sterile dissecting instruments (scissors, forceps).	2. Transfer dissected embryos into a new dish with PBS; remove heads and all internal organs.
3. Autoclaved 3-5 mm diameter glass beads and 1 to 2 inch stir bars.	3. Remove as much blood as possible by washing carcasses at least twice with 50 ml of PBS using 50-mL tubes.
4. 50-mL tubes (Falcon, cat. no. 2070).	4. Mince carcasses into small pieces with sterile scissors in minimal volume of PBS using 50-mL tube with the top cut off.
5. 100-mm Petri dishes, 150-mm cell culture plates.	5. Add 10 mL of Trypsin-EDTA; transfer into 50-mL tube. Put around 5 mL of glass beads and stir bar inside the tube. If solution becomes viscous, add 100 $\mu$ L of DNase per 10 mL.
6. DMEM plus 10% FBS.	6. Incubate at 37°C for 30 min with stirring.
7. 0.05% trypsin, 0.53 mM EDTA (e.g., Gibco, cat. no. 25300).	7. Add 10 mL of trypsin-EDTA, incubate at 37°C for another 30 min with stirring.
8. PBS without calcium and magnesium (e.g., Specialty Media, cat. no. BSS-1006-B).	8. Repeat step 7 one more time (final volume of 30 mL).
9. DNase I (Sigma, cat. no. D4527)	9. Decant cell suspension into two 50-mL tubes each containing 3 mL of FBS.
10. 1X freezing medium: 80% ES-DMEM, 10% FBS, 10% DMSO (prepared fresh prior to use and kept on ice).	10. Wash the tube twice with DMEM plus 10% FBS and add to the tubes with cell suspension.
11. Cryovials (e.g., Nalgene, cat. no. 5000-0012).	11. Centrifuge at 270g for 5 min.
	12. Resuspend the pellet in DMEM plus 10% FBS.
	13. Count viable nucleated cells using trypan blue (Flow Labs, cat. no. 16-910-49). Approximately $5 \times 10^7$ - $10^8$ cells could be expected from 10 fetuses.
	14. Plate $5 \times 10^6$ nucleated cells (approx from 1.25 embryo) per 150-mm dish, incubate at 37°C.
	15. Change the medium the next day.
	16. When confluent (in 2 to 3 days) trypsinize cells and split each plate onto 6 further plates.
	17. When these plates reach confluency, they can be frozen using 1X freezing media. Cells from each 150-mm plate are frozen in one cryovial in 1 mL of freezing media.
	18. Prepared Emfi stocks should be tested for mycoplasma, and mouse pathogens.

11. Cryovials (e.g., Nalgene, cat. no. 5000-0012).

18. Prepared Emfi stocks should be tested for mycoplasma, and mouse pathogens.

**Table 2**  
**Preparation of Mitomycin C-Treated Feeder Layers for ES Cell Culture**

Materials	Procedure
<ol style="list-style-type: none"><li>1. Frozen vials of Emfi stocks.</li><li>2. Cell culture plates.</li><li>3. DMEM plus 10% FBS.</li><li>4. 0.05% trypsin, 0.53 mM EDTA (e.g., Gibco cat. no. 25300).</li><li>5. PBS without calcium and magnesium (e.g., Specialty Media, cat. no. BSS-1006-B).</li><li>6. Mitomycin C (Sigma, cat. no. M0503). 1 mg/mL stock solution in PBS is stored light protected at 4°C for no longer than 2 wk.</li></ol> <p>As an alternative to mitomycin C treatment, <math>\gamma</math> irradiation at 6000–10,000 rads can be used for inhibition of cell growth.</p>	<ol style="list-style-type: none"><li>1. Thaw a frozen vial quickly at 37°C.</li><li>2. When ice crystals almost disappear, aseptically transfer cell suspension into a tube with DMEM plus 10% FBS. Centrifuge at 270g for 5 min, aspirate the supernatant.</li><li>3. Resuspend the pellet in DMEM plus 10% FBS, seed onto 5 to 6 150-mm plates (25 mL per plate) and incubate at 37°C.</li><li>4. When cells reach confluency (in about 3 d) they can either be:<ul style="list-style-type: none"><li>• split one more time before being treated with Mitomycin C, or</li><li>• treated with mitomycin C and used directly as feeders for ES cell culture, or</li><li>• treated with mitomycin C, frozen in cryovials and used as feeders later (alternative cost-efficient way for laboratories with small volume).</li></ul></li></ol> <p><b>Mitomycin C treatment</b></p> <ul style="list-style-type: none"><li>• Remove the medium from the confluent plate; add 10 mL DMEM plus 10% FBS and 100 <math>\mu</math>L of mitomycin C stock solution (1 mg/mL). Incubate the plate for 2 h at 37°C.</li><li>• Rinse twice with PBS, trypsinize as usual, and either freeze cells from each 150-mm plate in one cryovial for later use, or seed cells onto tissue culture plates for immediate use at appropriate cell densities and volumes (<math>2 \times 10^5</math> cells/mL).</li></ul> <p>One confluent 150-mm feeder plate can generate approximately the following number of feeder plates:</p> <ul style="list-style-type: none"><li>• 5 <math>\times</math> 100-mm plates (10 mL each);</li><li>• 12 <math>\times</math> 60-mm plates (5 mL each);</li><li>• 25 <math>\times</math> 35-mm plates (2 mL each);</li><li>• 25 <math>\times</math> 4-well plates or 4 to 5 <math>\times</math> 24-well plates (0.5 mL/well),</li><li>• 6 <math>\times</math> 96-well plates (200 <math>\mu</math>L /well)</li></ul> <p>Preferably, feeders are incubated overnight, or at least for a few hours before plating ES cells. The medium is changed to ES-DMEM prior to use. Mitomycin C treated feeders can be used within 7–10 d (medium is changed every 3 to 4 days).</p>

- d. 1 mM Sodium pyruvate (100X stock; Gibco, cat. no. 11360) stored at 4°C.
  - e. 0.1 mM  $\beta$ -mercaptoethanol (100X stock; Sigma, cat. no. M7522; or Specialty Media cat. no. ES-007-E) stored as aliquots at -20°C.
  - f. 2 mM L-Glutamine (100X stock; Gibco, cat. no. 25030; or Specialty Media, cat. no. TMS-002-C) stored as aliquots at -20°C.
  - g. 50 U/mL Penicillin and 50  $\mu$ g/mL streptomycin (100X Pen-Strep stock; Gibco, cat. no. 15140; or Specialty Media, cat. no. TMS-AB2-C) stored as aliquots at -20°C. Alternatively, 100X Pen-Strep-L-Glu combo available from Gibco (cat. no. 10378).
  - h. LIF (e.g., Chemicon International, ESCRO™ cat. no. ESG1107), stored as aliquots at -20°C (see Note 2).
2. *Feeder medium*: DMEM, 1X Pen-Strep, 10% FBS.
  3. *ES cell freezing medium*: freezing medium should be prepared fresh prior to use and kept on ice. We commonly use 22% FBS as a final concentration. It is possible to increase the concentration of FBS in a freezing media up to 40% (e.g., 2X ES cell-culture freezing medium from Specialty Media) for better recovery of small amount of cells in 96-well plates. 2X: 60% ES-DMEM, 20% FBS, 20% dimethyl sulfoxide (DMSO) (Sigma, cat. no. D5879). 1X: 80% ES-DMEM, 10% FBS, 10% DMSO.
  4. *0.1% Gelatin*: (Sigma, cat. no. G2500) 0.1% solution in water, autoclaved, and stored at 4°C. Alternatively ES cell-qualified 0.1% gelatin (Specialty Media, cat. no. ES-006-B).
  5. *Trypsin-EDTA*: 0.05% trypsin, 0.53 mM EDTA (Gibco, cat. no. 25300; Specialty Media, cat. no. SM-2002-C). 0.25% Trypsin, EDTA (Gibco cat. no. 25200 1 mM). Store at 4°C (main stock at -20°C).
  6. *Phosphate-buffered saline (PBS)* without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (e.g., Gibco cat. no. 10010; Specialty Media, cat. no. BSS-1006-B).
  7. *Selection reagents*: Geneticin® (G418) (Gibco, in liquid, cat. no. 10131, in dry powder form, cat. no. 11811; Sigma, cat. no. G9516), puromycin (Sigma, cat. no. P8833), hygromycin B (Calbiochem, cat. no. 400051). Working concentration for selection agents must be determined by killing curves. We routinely use 150–200  $\mu$ g/mL G418 for R1 cells.
  8. *Reagents for the lipofection*: LipofectAMINE™ (Gibco, cat. no. 18324-012 or LipofectAMINE™ 2000, cat. no. 11668-027); Opti-MEM® I reduced serum medium (Gibco, cat. no. 31985-062).

## 2.2. Isolation of DNA from 96-Well ES Cell Colonies

1. Lysis buffer: 10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.5% sarcosyl, 1 mg/mL proteinase K added before use.
2. NaCl-ethanol mixture 150  $\mu$ L of 5 M NaCl per 10 mL of cold 100% ethanol (prepared fresh).
3. Restriction digestion mixture (per well) 1X appropriate restriction buffer, 1 mM spermidine, 100  $\mu$ g/mL bovine serum albumin (BSA), 50–100  $\mu$ g/mL RNase A, 10–20 U of enzyme. Use 35–40  $\mu$ L per sample.

## 2.3. $\beta$ -Gal Staining of ES Cells and Postimplantation Stage Embryos

1. PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (e.g., Specialty Media, cat. no. BSS-1006-B).
2. Stock solutions: 10% Nonidet P-40 (NP40); 1% Na deoxycholate; 0.5 M EGTA; 1 M  $\text{MgCl}_2$ ; 0.5 M  $\text{K}_3[\text{Fe}(\text{CN})_6]$ , 0.5 M  $\text{K}_4[\text{Fe}(\text{CN})_6]$  (kept light protected at room temperature); 25–50 mg/mL 5-bromo-4-chloro-3-indolyl- $\beta$ -o-galactopyranoside (X-gal) in DMSO or dimethyl formamide (DMF) (e.g., Specialty Media, cat. no. BG-3-G) kept light-protected at -20°C.



3. Fix solution: 0.2% glutaraldehyde in PBS (for cells); 0.2% glutaraldehyde in PBS, containing 5 mM EGTA and 2 mM  $MgCl_2$  (for embryos). Glutaraldehyde (e.g., Sigma, cat. no. G6257) is added fresh prior to use. 2% Glutaraldehyde, 2 or 4% paraformaldehyde (PFA) are also used for fixation of embryos older than E12.5.
  4. Wash solution: PBS for cells; PBS, containing 0.01% Na-deoxycholate, 0.02% NP40, 5 mM EGTA, 2 mM  $MgCl_2$  for embryos.
  5. Stain solution: 1 mg/mL X-gal, 5 mM  $K_3[Fe(CN)_6]$ , 5 mM  $K_4[Fe(CN)_6]$  in wash solution (prepared fresh prior to use). Stain solution can be used several times if filtered after use and stored at  $-20^\circ C$ .
- Ready to use reagents for  $\beta$ -gal expression are available from Specialty Media (cat. nos. BG-1-C, BG-2-C, BG-4-C, BG-5-C, BG-6-B, BG-7-B, and BG-8-C).

## 2.4. Preimplantation Embryo Culture

### 2.4.1. Equipment

1. Stereomicroscope(s) (dissecting) with both transmitted and reflected lights or fiber optics light source with gooseneck. The use of two microscopes is convenient for 2-cell stage embryo fusion and embryo transfer into pseudopregnant females, however, one microscope is also enough. We find that the frosted glass, instead of the more common, transparent glass in the base of the microscope, gives better view of the zonae pellucidae of preimplantation stage embryos necessary for zona removal.
2. Humidified incubator ( $37^\circ C$ , 5%  $CO_2$ ).
3. Sterile Petri dishes of different size (60, 100 mm), organ culture dishes (Falcon, cat. no. 3037). We find the plastic of 35-mm Easy Grip Falcon (cat. no. 3001) tissue culture plates to be best suited for making depressions for aggregations.
4. Sterile 1-mL syringes, 26-gauge 1/2 inch long needles (26G1/2), 30G1/2 needles. To make a flushing needle, the sharp tip of 30G1/2 needle is first cut off and then polished on a sharpening stone or sand paper. The flushing needle is flushed with 70% ethanol before and after use.
5. Bunsen or alcohol burner.
6. Pasteur pipets that are drawn by hand over a flame and broken to produce pipets for embryo manipulation, with a diameter of a capillary slightly larger than an embryo. It is important to flame-polish the tip of the pipet as the embryos without zonae are easily damaged. Embryo manipulating pipets are connected through a latex tubing to an aspirator mouthpiece (HPI Hospital Products Med. Tech., cat. no. 1501P-B4036-2). Such a mouth-controlled pipet is used for all embryo manipulations and embryo transfer. Alternatively, a finger-controlled pipet (small piece of tubing closed at one end or small bulb connected to the drawn capillary) is used for embryo manipulations.
7. Surgical instruments (e.g., Fine Scientific Tools [FST]): sharp fine-pointed scissors, fine forceps (e.g., Dumont cat. no. 5 or ss/mc), straight or curved blunt forceps with serrated tips, forceps with  $1 \times 2$  teeth, serrefine (FST, cat. no. 18050-28); wound clips and Autoclip applier (Clay Adams B-D 7631 and B-D 763007).
8. Cell-fusion instrument CF-150B, aggregation needle DN-09 (BLS Ltd, Hungary, [www.bls-ltd.com], e-mail: bls@euroweb.hu)

### 2.4.2. Mouse Stock

C57 Bl/6 mice are the most common strain used as donors of host embryos for chimera production by blastocyst injection of different ES cell lines. In our facility, random-bred ICR (CD-1) mice (available from Charles River Laboratories, Harlan Sprague Dawley, or Taconic) have been successfully used for many years as both

donors of host embryos and recipients of manipulated embryos. The mouse colony necessary for the creation of chimeras should contain a stock of females, stud males, and vasectomized males. The details of maintenance of such a colony as well as all procedures involved in the production of superovulated and pseudopregnant animals are described in multiple publications (17,19).

#### 2.4.3. Preimplantation Embryo Culture Media and Reagents

Since embryos are cultured for 24 or even 48 h in aggregation experiments, the quality of culture conditions is more critical than in blastocyst injection. Embryo culture media is commercially available (e.g., from Specialty Media: KSOM medium, cat. no. MR-023-D, M2 medium, cat. no. MR-015-D). However, as culture media can not be used for longer than 2, or a maximum of 3 wk, it is often necessary to prepare media from scratch or from stocks, as described previously (19). We keep all our concentrated stocks (see Tables 3 and 4) at  $-70^{\circ}\text{C}$  for a few months. BSA (Sigma, cat. no. A3311) is kept desiccated at  $4^{\circ}\text{C}$  (see Note 3).

1. M2 is a HEPES-buffered media that is used during embryo collection and other manipulations in room atmosphere (fusion, zonae removal). Filtered aliquots are stored at  $4^{\circ}\text{C}$  and brought up to room temperature prior to use. Embryos should not be kept in M2 for prolonged periods of time and are rinsed well with a few drops of equilibrated KSOM media before being placed into the incubator.
2. KSOM is a bicarbonate-buffered media used for embryo culture and was developed through a simplex optimization procedure by Lawitts and Biggers (20). It may also be supplemented with both essential and nonessential amino acids, which were shown to improve the development in vitro (21). Filtered aliquots are stored at  $4^{\circ}\text{C}$  and equilibrated by placing the tube, a 1-mL syringe, or the culture dish containing KSOM in the incubator well in advance before use.
3. Embryo-tested light mineral oil (eg., Sigma, cat. no. M8410).
4. Acidic tyrode solution (Sigma, cat. no. T1788) for removing zonae. Main stock aliquots are stored at  $-20^{\circ}\text{C}$ . An aliquot is thawed and kept at  $4^{\circ}\text{C}$  when needed, and it is brought to room temperature prior to use.
5. 0.3 mol/L Mannitol (Sigma, cat. no. M4125) in ultrapure water containing 0.3% BSA for embryo fusion. Aliquots are stored at  $-20^{\circ}\text{C}$ . A freshly thawed aliquot is used for fusion, and the unused portion is discarded.

### 3. Methods

#### 3.1. ES Cell Culture

##### 3.1.1. Passage of ES Cells

See Note 4 for the general considerations on ES cell maintenance.

1. Prepare the necessary number of gelatinized plates by coating them with 0.1% gelatin: rinse the plate with 0.1% gelatin solution covering the surface (5 mL/100-mm plate), leave for a few minutes, aspirate, and allow to dry for a few minutes, add ES-DMEM, and place in the incubator. Alternatively, replace the media on the appropriate number of prepared feeder plates to ES-DMEM.
2. Aspirate the growth medium, rinse twice with PBS, add trypsin (1.5–2 mL/100-mm, 1–1.5 mL/60-mm, 0.5 mL/35-mm dish, 0.25–0.3 mL/well for 4- or 24-well plates), place in the incubator for 5 min.

**Table 3**  
**Preparation of M2 Medium from Concentrated Stocks**

Component	M2 Medium		Concentrated stock	Stock volume for 100 mL
	Final concentration mM	g/L		
			<i>A (10X)</i> g/100 mL	10 mL
NaCl	94.66	5.534	5.534	
KCl	4.78	0.356	0.356	
KH <sub>2</sub> PO <sub>4</sub>	1.19	0.162	0.162	
MgSO <sub>4</sub> × 7 H <sub>2</sub> O	1.19	0.293	0.293	
Glucose	5.56	1.000	1.000	
Penicillin G		0.060	0.060	
Streptomycin		0.050	0.050	
Sodium lactate	23.28	2.610 or 4.349 g of 60% syrup	2.610 or 4.349 g of 60% syrup	
			<i>B (10X)</i> g/100 mL	1.6 mL
NaHCO <sub>3</sub>	4.15	0.349	2.101	
Phenol Red			0.001 or 0.01 mL of 0.5% solution	
			<i>C (100X)</i> g/10mL	1 mL
Na pyruvate	0.33	0.036	0.036	
			<i>D (100X)</i> g/10 mL	1 mL
CaCl <sub>2</sub> × 2H <sub>2</sub> O	1.71	0.25	0.25	
			<i>E (10X)</i> g/100 mL	8.4 mL
HEPES	20.85	4.969	5.958	
Phenol Red			0.001 or 0.01 mL of 0.5% solution	
BSA (Sigma, cat. no. A3311)		4.000		400 mg

If necessary, pH of M2 is adjusted to 7.2–7.4 with 0.2 N NaOH. Osmolarity of M2 should be 285–287 mOsm. Media is filter-sterilized, aliquoted in polypropylene tubes, and stored at 4°C.

3. Swirl the plate to detach clumps from the bottom of the plate, pipet the cells gently to break the clumps (optional). Add an equal volume of ES-DMEM to neutralize trypsin, pipet up and down several times, transfer the suspension into a 12-mL tube. Pellet the cells by low-speed centrifugation (270g) for 5 min at room temperature.
4. Aspirate the supernatant, add 1 drop of PBS or ES-DMEM to the pellet, flick the tube to resuspend the cells before adding ES-DMEM (optional).
5. Add 5–7 mL of ES-DMEM to the tube, pipet gently to mix well, and split the contents at a 1:5 or 1:7 ratio into the new plates containing a sufficient volume of medium (5 mL/60-mm, 10 mL/100-mm plate). About  $1 \times 10^6$  cells onto 60-mm plate,  $2 \times 10^6$  cells onto 100-mm plate.
6. Change the medium the next day and split every second day as described.

**Table 4**  
**Preparation of KSOM Medium from Concentrated Stocks**

KSOM Medium				
Component	Final concentration mM	g/L	Concentrated stock	Stock volume for 100 mL
<i>A' (10X)</i>				
			<i>g/100 mL</i>	<i>10 mL</i>
NaCl	95.00	5.55	5.55	
KCl	2.50	0.186	0.186	
KH <sub>2</sub> PO <sub>4</sub>	0.35	0.0476	0.0476	
MgSO <sub>4</sub> × 7 H <sub>2</sub> O	0.20	0.0493	0.0493	
Glucose	0.20	0.036	0.036	
Penicillin G		0.060	0.060	
Streptomycin		0.050	0.050	
Sodium lactate	10.0	1.12 or 1.87 g of 60% syrup	1.12 or 1.87 g of 60% syrup	
<i>B' (10X)</i>				
			<i>g/100 mL</i>	<i>10 mL</i>
NaHCO <sub>3</sub>	25.00	2.10	2.10	
Phenol Red			0.001 or 0.01 mL of 0.5% solution	
<i>C' (100X)</i>				
			<i>g/10 mL</i>	<i>1 mL</i>
Na pyruvate	0.20	0.022	0.022	
<i>D (100X)</i>				
			<i>g/10 mL</i>	<i>1 mL</i>
CaCl <sub>2</sub> × 2 H <sub>2</sub> O	1.71	0.25	0.25	
<i>F (1000X)</i>				
			<i>g/10 mL</i>	<i>0.1 mL</i>
EDTA (Na disodium salt)	0.01	0.0038	0.038	
<i>G (200X)</i>				
			<i>200 mM</i>	<i>0.5 mL</i>
L-Glutamine (Gibco, cat. no. 25030)	1.00	0.146	Liquid form	
BSA (Sigma, cat. no. A3311)		1.000		100 mg

Media is filter-sterilized, aliquoted in polypropylene tubes and stored at 4°C. Osmolarity of KSOM should be 256 mOsm. Optimally, KSOM media is gassed with 5% CO<sub>2</sub> in air before storage. KSOM media needs to be equilibrated in 5% CO<sub>2</sub> prior to use for embryo culture.

### 3.1.2. Freezing and Thawing of ES Cells in Cryovials

Usually ES cells are frozen at about  $5 \times 10^6$  cells/mL of 1X freezing media (approx 4 vials from 100-mm dish).

1. Change growth media 2 to 3 h before freezing the cells (optional).
2. Freshly prepare 2X or 1X freezing media (see Subheading 2.1.3.).

- 3 Harvest the cells in a 12-mL tube containing ES-DMEM as described in **Subheading 3.1.1**. Pellet the cells at 270g for 5 min at room temperature.
- 4 Remove the supernatant, resuspend the cells gently in half of the final volume required using ES-DMEM, gradually add an equal volume of 2X freezing medium while shaking the tube, and mix by pipetting up and down several times. Alternatively, gently resuspend the pellet in 1X freezing media.
- 5 Quickly aliquot 1 mL of the cell suspension into labeled cryovials and immediately place them in a precooled styrofoam box that will allow them to cool down gradually. Alternatively, isopropanol containers purchased from a number of manufacturers (e.g., Nalgene, cat. no. 5100-0001) can be used.
- 6 Immediately place the container in a  $-70^{\circ}\text{C}$  freezer for 1 to 2 d, then transfer cryovials into a liquid nitrogen tank for long-term storage.

A vial, frozen in such a way, can be thawed onto a 60-mm plate. Freezing and thawing are usually counted as one passage.

- 1 Thaw the vial by quickly warming it at  $37^{\circ}\text{C}$ .
- 2 When ice crystals almost disappear, aseptically transfer the cell suspension into a 12-mL tube using the pipet filled with ES-DMEM to slowly dilute DMSO.
- 3 Pellet the cells at 270g for 5 min and aspirate the supernatant.
- 4 Resuspend the pellet in fresh ES-DMEM, plate on a gelatinized or feeder plate, gently swirl the plate bidirectionally to evenly distribute the cells, and place in the incubator.
- 5 The next day, remove floating dead cells and change the media. If the correct procedure was used, cells should be ready for passage in 2 to 3 d.

### 3.2. Introduction of DNA into ES Cells

Electroporation is the most common way of in vitro introduction for both stable integration and transient expression in ES cells. Recently, we have successfully used lipofection for transient transfection of Cre recombinase into single-copy integrants. It is important to test the excision of the STOP region in vitro before in vivo experiments. In our example, ES cells become neo-sensitive and lose *lacZ* activity after Cre excision (see Fig. 1).

#### 3.2.1. Electroporation of ES Cells

Cells are routinely passaged 2 d prior to electroporation. Usually one 10-cm plate at approx 80% confluency ( $15\text{--}20 \times 10^6$  cells/mL) will provide enough cells for 1 to 2 electroporations. We regularly electroporate 20–40  $\mu\text{g}$  of DNA into cells at the density of  $7 \times 10^6$  cells/mL. Vector DNA is linearized by restriction enzyme digestion, extracted twice with phenol:chloroform (optional), ethanol-precipitated, washed twice in 0.5–1 mL of 70% ethanol, and resuspended in sterile  $0.1 \times$  Tris-EDTA (TE), PBS, or water at a concentration of 1  $\mu\text{g}/\mu\text{L}$ . For transient expression of Cre recombinase, circular plasmid is used for electroporation. Electroporated cells are then plated very sparsely (approx 1000 cells/100-mm dish).

1. Change medium on ES cells at least 2 h prior to electroporation.
2. Switch on the electroporation apparatus and set up conditions in advance. We routinely use 250 V, 500  $\mu\text{F}$  for the Bio-Rad GenePulser<sup>®</sup> (cat. no. 165-2106) and capacitance extender (cat. no. 165-2108).
3. Harvest cells as described in **Subheading 3.1.1**. It is critical to get a single-cell suspension. Pool the cells from all dishes into one tube.

4. Resuspend the pellet in a minimal volume of ice-cold PBS (about 1 mL/100-mm plate). Determine the cell density with a hemocytometer and dilute with a volume of PBS that will give the required density of a cell suspension (see above). Keep the suspension on ice. Recently, we found that ES cell electroporation buffer available from Specialty Media (cat. no. ES-003-D), used instead of PBS in this step, gives significantly better recovery of electroporated ES cells.
5. Gently mix 0.8 mL of the ES cell suspension and 20–40 µg of DNA in a precooled electroporation cuvette.
6. Electroporate the cells, then place the cuvettes on ice for 20 min (optional).
7. Prepare the appropriate number of gelatinized plates. The number of plates will depend on the cells' survival, specific vector, selection approach, and desired density of colonies. We routinely plate cells from one cuvette onto 1.5–2 × 100-mm plates.
8. Transfer electroporated cells from the cuvette into the tube with the appropriate volume of ES-DMEM (15–20 mL/cuvette). Cells from several cuvettes can be pooled into one tube and gently mixed by pipetting into a uniform suspension. Transfer cell suspension onto gelatinized plates, swirl bidirectionally to evenly distribute cells across the surface. Incubate overnight and change the medium the next day.
9. Start drug selection 24–48 h after electroporation. Change the selection media every day for the first few days, then every other day. Continue the selection until colonies become apparent and ready to pick (*see Subheading 3.3.1.*) i.e., visible to the naked eye, it usually takes 6–10 d.

### 3.2.2. Lipofection for Transient Expression of Transgenes

Based on Gibco protocols for LipofectAMINE, cat. nos. 18324-012 and 11668-027.

1. Plate ES cells to a 35-mm or 6-well gelatinized dish ( $2 \times 10^5$  cells/dish/well) using ES-DMEM.
2. Incubate overnight, the cells should reach 30–40% confluency.
3. Prepare the following solutions in 5-mL polystyrene tubes (Falcon, cat. no. 352058). It is possible to use serum-free or reduced serum ES-DMEM instead of OPTI-MEM. Antibacterial agents should not be present in either media during the transfection.  
Solution A: For each well of a 6-well plate or 35-mm dish, mix 1 µg of circular plasmid containing an expression vector DNA with 300 µL of OPTI-MEM reduced serum medium (Gibco, cat. no. 31985).  
Solution B: For each well of a 6-well plate or 35-mm dish, mix 8 µL of Lipofectamine reagent (LF) with 300 µL of OPTI-MEM (*see Note 5*).
4. Combine necessary volumes of solutions A and B, mix gently, and incubate at room temperature for 20 min.
5. Place the solution (500 µL/well/dish) onto ES cells rinsed with OPTI-MEM. Incubate for 5 h at 37°C.
6. Replace the media with drug-free ES-DMEM or proceed with selection.

### 3.3. Growing Drug-Resistant ES Cell Clones

#### 3.3.1. Picking ES Cell Clones into 96-Well Plates

Well-separated colonies of similar size with a defined perimeter and a compact center with undistinguishable individual cells should be picked. Large colonies and those with large distinguishable cells are differentiating and should be avoided if possible. Colonies can either be picked with the naked eye or by using a dissecting

microscope with transmitted light. They can also be circled on the bottom of the plate with a marker for easier visualization.

1. Using the multichannel pipettor, prepare the appropriate number of gelatinized flat-bottom 96-well plates. Aliquot 30–50  $\mu$ L of trypsin into the wells of a V-bottom 96-well plate.
2. Aspirate growth media from the plate with ES colonies, rinse it twice with PBS, add 6–8 mL of PBS to completely cover the dish
3. Using a drawn Pasteur pipet or a Gilson P20 or P200 set at 15  $\mu$ L, carefully dislodge the colony from the dish and pull it into the pipet tip with as little volume of PBS as possible (usually 3–5  $\mu$ L).
4. Transfer each individual colony in a minimal volume of PBS into one of the wells of a V-bottom 96-well plate containing trypsin.
5. Using a new tip for each colony, proceed with the rest. This process should not take longer than 30–60 min, 48 or 96 colonies should be picked at a time, depending on the picking speed.
6. Place a 96-well plate in a 37°C incubator for 10 min.
7. Working row by row with a multichannel pipettor, add 50–70  $\mu$ L of selective ES-DMEM to each well with trypsin (to 100  $\mu$ L/well). Gently pipet up and down several times to disaggregate the cells. Transfer the suspension to the equivalent row of gelatinized plates. Alternatively, add ES-DMEM to all the wells to neutralize the trypsin first, and then proceed with pipetting and transfer.
8. Using the multichannel pipettor, wash each well of the V-bottom plate with another 100  $\mu$ L of medium, and transfer the volume to the equivalent row in a flat-bottom 96-well gelatinized plate (to total vol of 200  $\mu$ L/well). Place the plate into the 37°C incubator.
9. Change the media daily until the cells are ready for passage (80% confluency).

### 3.3.2. Passage of ES Cells in 96-Well Plates

Optimally, 3 or 4 d after picking colonies into the 96-well plates, the cells reach the density required for passage (*see Note 6*). Replica plates are used for the preparation of DNA for screening, X-gal staining, and for creating frozen stocks. It is generally recommended to have two confluent replica plates for DNA preparation and one or two replica plates to keep as a frozen stock. It is also important to identify strong overall expresser clones using the reporter system of choice (X-gal staining in our example). The wells equivalent to such identified strong expresser clones are used for later isolation of DNA for Southern blotting to test single site-copy integration. Confirmed single-copy integrants are thawed and expanded for further *in vitro* and *in vivo* analysis.

1. Prepare the required number of gelatinized flat 96-well plates. Add 150  $\mu$ L of ES-DMEM per well and place in a 37°C incubator until necessary.
2. Aspirate the medium from the plate to be split and wash twice with 200  $\mu$ L of PBS using the multichannel pipettor.
3. Add 50  $\mu$ L of trypsin per well. Incubate at 37°C for 10 min. The cells should detach with gentle tapping on the plate.
4. Add 50  $\mu$ L of medium into each of the wells to stop trypsinization. Pipet up and down at least five times to mix well. Working row by row, transfer the cell suspension into 2 or 3 new gelatinized plates containing ES-DMEM. Place in the 37°C incubator and change the media the next day. Alternatively, one half of such a cell suspension can be frozen right away in one new V-bottom or flat 96-well plate containing 2X freezing media (*see*

**Subheading 3.3.3.**) At least one more passage is required to create two replica plates for DNA preparation and one for X-gal staining in addition to one or two frozen plates.

### 3.3.3. Freezing and Thawing of ES Cells in 96-Well Plates

1. Freshly prepare 2X cell freezing media and keep it on ice.
2. Trypsinize the cells of an 80% confluent 96-well plate as described in **Subheading 3.3.2.** The final volume of the cell suspension is 100  $\mu$ L/well.
3. Working quickly on ice, aliquot 100  $\mu$ L of 2X freezing media into each well. Pipet the cells up and down several times to get a homogeneous suspension. Alternatively, transfer the cell suspension into the new V-bottom 96-well plate, containing cold 2X freezing media.
4. Add 50  $\mu$ L of cold sterile mineral oil (e.g., Sigma, cat. no. M8410; or Specialty Media, cat. no. ES-005-C) to each well on top of the cell suspension.
5. Wrap the plates in parafilm and foil (the latter is optional). Place in a precooled styrofoam box, and store in a  $-70^{\circ}\text{C}$  freezer, preferably not longer than 2 mo, until ready for thawing and expansion.

The first frozen plates are considered as master plates. The unfrozen replica plates are used for further characterization of the clones. After strong expresser and single-copy integrant clones have been identified, the frozen stock can be thawed and expanded for further analysis as described below.

1. Prepare the necessary number of 4- or 24-well feeder plates containing ES-DMEM.
2. Remove the plate containing the identified clones from the freezer. Unwrap the plate and place in the incubator to thaw.
3. When ice crystals almost disappear, wipe the outside of the plate with 70% ethanol.
4. Transfer the content of the well into the wells of prepared feeders plate.
5. Rinse the original wells of the 96-well plate with more ES-DMEM and transfer to the same wells. Change the media after overnight culture and daily.
6. Passage the cells when they reach 70–80% confluency to a larger plate (i.e., 35 mm). If cells do not reach confluency in a few days, but form few colonies in a well, they can be trypsinized, broken into smaller cell clumps, and plated on the same plate (*see Note 6*).
7. Passage every other day, freeze the cells in vials as described (*see Subheading 3.1.1. and 3.1.2.*). Use them for in vitro tests (i.e., transient expression of Cre recombinase by electroporation or lipofection).

### 3.4. $\beta$ -Gal Staining of ES Cells and Postimplantation Stage Embryos

*lacZ* gene expression is detected by the enzymatic activity of the gene product  $\beta$ -gal in both embryos and cells. Generally, the staining becomes visible after a 15-min or an overnight incubation, depending on the level of expression.

1. Wash cells or embryos twice in PBS.
2. Add freshly prepared fixative solution to completely cover the cells or embryos, incubate at room temperature for 5–7 min (for cells), 5 min (for up to E9.5 embryos), and 15–20 min (for up to E12.5 embryos) (*see Note 7*).
3. Wash three times with PBS (cells) or wash buffer (embryos).
4. Replace the wash buffer with X-gal stain to completely cover the cells or embryos and incubate at  $37^{\circ}\text{C}$  light protected with optional shaking.
5. Replace the X-gal stain with PBS (cells) or wash buffer (embryos) and store at  $4^{\circ}\text{C}$ . For longer storage, embryos can be refixed in a fresh 4% solution of PFA or formaldehyde.



### 3.5. DNA Isolation from ES Clones in 96-Well Plates

This procedure was established by Ramirez-Solis et al. (22) and allows cell lysis, DNA precipitation, and the restriction digestion in the original 96-well plate in which ES cells were growing. Usually two replica plates are used for DNA isolation, then one is processed for Southern blot analysis, leaving the second plate as a back up. The cells should be lysed, and genomic DNA isolated from them when the majority of clones are confluent. The yellow color of the media within 24 h of its change indicates cell confluency.

1. Aspirate media from each well, wash twice with PBS.
2. Add 50  $\mu$ L of lysis buffer to each well.
3. Incubate the plates overnight at 55°C in a humid atmosphere (wrap the plates sealed with the parafilm with wet paper towels and place in a plastic container or sealed plastic bag).
4. The next day, add 100  $\mu$ L of cold NaCl-ethanol mixture to each well.
5. Leave the plate undisturbed at room temperature for 30–60 min (or more if necessary) until the precipitated DNA attached to the dish is visible against a dark background.
6. Gently invert the plate on the paper towel to drain the liquid.
7. Rinse 3 times with 150–200  $\mu$ L of 70% ethanol per well, inverting the plate each time. After this step, DNA can be stored in 70% ethanol at –20°C.
8. Invert the plate after the final wash and allow to air-dry for 10–15 min (it is important for all the ethanol to dry out).
9. Add 34–40  $\mu$ L of restriction digest mixture per well, mix, seal the plate, and incubate overnight at 37°C in a humid atmosphere.
10. Proceed with Southern blot analysis.

### 3.6. Introduction of ES Cells into Mice

As our ES cell line of choice is R1(18), we use aggregation of ES cells with cleavage stage host ICR embryos to create the chimeras (23), although blastocyst injections may be used as well. Concerning the conditional transgenic lines, a faster way to test the in vivo expression of transgenes introduced into several candidate ES cell clones is by analyzing chimeras produced by aggregation with tetraploid or diploid embryos (17). Clones that give the expected results are then chosen for germline transmission. After successful germline transmission, conditional transgenic animals can be crossed with switch transgenic lines, where Cre recombinase is driven by lineage-specific promoters.

#### 3.6.1. Preparation of ES Cells for Aggregation

It is important to maintain optimal culture conditions for all ES cell cultures, but particularly for ES cell clones to be introduced into mice (see Note 4). At least one passage on a gelatinized plate is required before aggregation. Sparser than usual, passage 1 or 2 d before aggregation produces the colonies of 8–15 cells that are lifted by gentle trypsinization.

1. Three or four days prior to aggregation, thaw a vial of ES cells or passage as described above. Change the media the next day.
2. One or two days prior to aggregation, trypsinize ES cells as described in Subheading 3.1.1. and ensure a single-cell suspension. Twenty-four hours growth is enough for most clones, but 48 h are necessary for slower growing clones.

3. Resuspend the pellet in ES-DMEM. Leave the tube undisturbed for a few minutes to allow for large clumps and feeders to settle.
4. Seed the cell suspension from the top portion onto a few gelatinized plates using different dilutions (e.g., 1:10 to 1:50).
5. On the day of aggregation, after preparation of the embryos (*see Subheading 3.6.3.*), wash the cells first with PBS, then with trypsin (optional).
6. Add minimal amount of trypsin to just cover cells (0.5 mL/60-mm plate) and place in the incubator for 1 to 2 min or leave at room temperature until colonies start to detach from the plate.
7. Watch under the microscope for colonies to detach from the plate, gently swirl the plate, or tap at the microscope stage. Do not over-trypsinize, as cells will become sticky and hard to manipulate. Add the required volume of ES-DMEM to the plate. Do not pipet. ES cells are now ready for aggregation during the next hour or two. Keep the plate at room temperature, as cells will start attaching to the plate in the incubator (*see Note 8*).

### 3.6.2. Recovery of 8- and 2-Cell Stage Embryos

It is preferable to use only noncompact 8-cell stage embryos for aggregation. However, we routinely use all embryos with intact blastomeres, from the 8-cell to morula stage, collected at 2.5 d postcoitum (dpc). Two-cell stage embryos, collected at 1.5 dpc, are used for production of tetraploid embryos (*see Subheading 3.6.4.*). The recovery procedure for 2-cell stage embryos is essentially the same.

1. Prepare the culture plates using KSOM media (organ culture or microdrops overlaid with mineral oil). Place the tube or the syringe with KSOM into the incubator.
2. Dissect the oviducts from 2.5 dpc pregnant females, leaving the upper part of the uterus attached, and place in the drop of M2.
3. Transfer one oviduct into a small drop of M2.
4. Using the dissecting microscope, insert the flushing needle attached to a 1-mL syringe filled with M2 into the infundibulum. The use of fine forceps helps to place the needle in the right position. Flush M2 media through the oviduct and observe its swelling.
5. Proceed with the remaining oviducts, keeping the time of manipulations in M2 minimal.
6. Collect embryos into a fresh M2 drop using embryo-manipulating mouth- or finger-controlled drawn Pasteur pipet. Wash them through several M2 drops to get rid of debris.
7. Wash embryos through few drops of equilibrated KSOM and place them in the culture dish.

### 3.6.3. Aggregation of ES Cells with Cleavage Stage Embryos

#### 3.6.3.1. PREPARATION OF THE AGGREGATION PLATE

1. Using a 1-mL syringe filled with KSOM media, place microdrops (approx 3 mm in diameter) on a 35-mm dish. We usually place 2 rows of 4 to 5 drops in the middle of the plate and 2 more rows of 3 drops on each side. Cover with mineral oil.
2. Wipe the aggregation needle with 70% ethanol. Press the needle into the plastic, make a slight circular movement (*see Note 9*). We usually make 6 depressions per KSOM drop. Three or 6 drops on the sides are left without depressions. They are used for the final selection of ES cell clumps. The plate holds 40–60 aggregates.
3. The plate is placed into the incubator to allow good equilibration of the media.

#### 3.6.3.2. ZONA REMOVAL

In order to allow the attachment of ES cells to the blastomeres of the embryos as an obvious precondition of aggregation, the zonae pellucidae of the embryos need to

be removed. We use acidic Tyrode solution to dissolve this glycoprotein membrane. Since the acid diluted with buffered solution will not work as efficiently and the acid transferred into culture media will damage the embryos, it is important to transfer a minimal amount of solutions between drops and use multiple washes. The number of embryos manipulated at a time depends on the speed of manipulations.

1. Place M2 and acid Tyrode's drops into a 100-mm Petri dish. The temperature of the acid tyrode should not exceed room temperature.
2. Pick 20–50 embryos with a minimal volume of media and wash them through one acid drop. Transfer embryos to a fresh drop of acid. Move them by pipetting and observe zona dissolution.
3. As soon as the zonae dissolve, immediately transfer the embryos with a minimal volume of acid to a M2 drop. Rinse through several M2 drops. Do not allow the embryos to touch each other. Proceed with the remaining embryos.
4. Wash the embryos through several drops of equilibrated KSOM and place them into the aggregation plates.

### 3.6.3.3. ASSEMBLY OF AGGREGATES

We aggregate an ES cell clump of 8–15 cells with one diploid embryo. Two tetraploid embryos are also usually aggregated with such a clump of ES cells (*see Subheading 3.6.3.4.*). It is possible to use one tetraploid embryo for aggregation, but it might be necessary to culture aggregates for one more night to ensure blastocyst formation. Aggregates are assembled in either of two ways: (1) the embryo is first placed into the depression, and the clump of ES cells is placed next to it, or (2) the clumps of ES cells are first distributed into depressions, and then the embryos are placed next to the clumps. Both ways work equally well.

1. After zona removal, embryos are placed into the aggregation plates inside the depressions or beside them, making sure they are not touching each other. The plates are kept in the incubator until the ES cells are ready.
2. Prepare ES cells for aggregation as described in the **Subheading 3.6.1.**
3. Under the dissecting microscope, choose a number of clumps of required size and transfer them into the microdrops, not containing depressions, for final selection.
4. Select a few clumps of 8–15 cells and carefully transfer them individually into the depressions, next to the embryos. Alternatively, distribute clumps into all depressions in a plate, then place the embryos next to each clump.
5. Assemble all the aggregates in this manner, check the plate to ensure that all ES cell clumps are in contact with embryos, and incubate overnight. The next day, the majority of aggregates will be blastocysts or late morulae. They can be transferred into 2.5 dpc pseudopregnant females as described previously (17,19) (*see Note 10*).

### 3.6.4. Generation of Tetraploid Embryos

The electrofusion of blastomeres of 2-cell stage embryos for the generation of tetraploid embryos was first developed by Kubiak and Tarkowski (24). Most tetraploid embryos die shortly after implantation (25), but when aggregated with diploid embryos, they can contribute to the primitive endoderm and trophoctoderm lineages and are excluded from the primitive ectoderm lineage (26). When the tetraploid embryos are aggregated with ES cells, the resulting fetuses are ES cell derived, as ES cells do not contribute to trophectoderm and primitive endoderm. Thus, ES cell and tetraploid

components complement each other. Therefore, the method of ES cell  $\leftrightarrow$  tetraploid embryo aggregation can be used, for example, as a rapid test for the developmental potential as well as for the transgene expression of an ES cell clone. The application of ES cell  $\leftrightarrow$  tertraploid embryo chimeras is, however, much broader (*see ref. 27* for review).

1. Prepare the culture plate (microdrops of KSOM overlaid with mineral oil). Thaw a frozen aliquot of mannitol.
2. Collect 2-cell stage embryos as described in **Subheading 3.6.2**.
3. Turn on the cell-fusion instrument and set up the parameters. The fusion of blastomeres of 2-cell stage embryos occurs when a DC electric pulse is applied perpendicular to the plane of the blastomeres' contact. We apply one or two pulses of 30 V and 40  $\mu$ s for the fusion in nonelectrolyte solution (mannitol) using the CF-150B cell-fusion instrument from BLS Ltd. The adjustable 1 MHz AC field (1 to 2 V) allow the orientation of 2-cell stage embryos in the electrode chamber (distance between electrodes is 250  $\mu$ m). The actual parameters might vary and need to be determined in a pilot experiment. The goal is to reach 90% fusion in 30–60 min without embryo lysis.
4. Place an electrode chamber connected to the pulse generator into the 100-mm Petri dish. Use the same dish for embryo washes; alternatively, use another plate on a second microscope.
5. Place two large drops of M2 and a drop of mannitol solution in the dish. Place the mannitol drop over the electrode chamber.
6. Pick 25–30 embryos and rinse them well with mannitol by quickly pipetting up and down. Place the embryos between the electrodes, spacing them from each other (*see Note 11*). Manually move the embryos that are not oriented.
7. When all the embryos are properly oriented, push the trigger pulse.
8. Immediately transfer the embryos into an M2 drop. Wash the embryos through a few drops of equilibrated KSOM. Place them into the culture plate in the incubator.
9. Proceed with the rest of the embryos. The mannitol drop over the electrode chamber should not be used for longer than 15 min and should be replaced with a fresh one after that time. The number of embryos handled in 15 min depends on the speed of manipulations.
10. It is very important to select perfectly fused tetraploid embryos 30–60 min after application of the pulse and transfer them into a fresh drop. Since embryos are recovered at the late 2-cell stage, the second mitotic division is expected soon after fusion. If not checked in time, fused and cleaved tetraploid embryos could be confused with non-fused diploid 2-cell stage embryos. Under optimal conditions, around 90–95% of embryos should fuse.
11. After overnight incubation, 3- to 4-cell stage embryos are used for sandwich aggregation as described in the **Subheading 3.6.3**.

#### 4. Notes

1. The quality of FBS is the most important factor for successful ES cell culture. ES cell qualified pretested serum is available from some suppliers (e.g., Gibco and Specialty Media). It is still recommended to test different lots of serum from different companies before ordering large quantities (*see ref. 17* for testing serum batches protocol). It is also possible to use Knockout Serum Replacement (SR)<sup>TM</sup> from Gibco (cat. no. 10828) in combination with regular DMEM or Knockout D-MEM (Gibco, cat. no. 10829) for ES cell culture. However, in this case, ES cells need to be grown on feeders all the time.
2. It is possible to purify LIF from bacteria transfected with recombinant LIF constructs (28). Each new batch of LIF should be tested for the final concentration necessary to maintain pluripotency of ES cells. For R1 ES cells, we use final concentration of 1000 U/mL.

3. The quality of water is possibly even more critical for embryo culture than for ES cell media (*see Subheading 2.1.3.*). Disposable plasticware is highly recommended, or if clean glassware is used, it should never be exposed to detergent or organic solvents. All chemicals should be of highest grade, or embryo-tested (available from Sigma) and used only for media preparation. Embryos are cultured in organ culture dishes or in microdrops of KSOM media covered with embryo-tested light mineral oil (e.g., Sigma, cat. no. M8410). A majority of E0.5 dpc embryos reaching blastocyst stage demonstrates the optimal culture conditions. The minimal time between sacrificing the embryo donors and putting the embryos in the culture dish and the proper removal of debris after flushing also contributes to successful embryo culture.
4. It is important to carefully follow the ES cell culture protocol to maintain their pluripotency and ability to contribute to the germline. Typically, ES cells are kept at relatively high density and should be passaged when they reach a subconfluent state of 70–80%, i.e., tightly packed colonies almost touch each other. Media should be changed every day. For regular maintenance ES cells should never be seeded too sparsely (when 4 to 5 d is required to reach subconfluency), nor should they grow past 90% confluency before passage. Both conditions induce cell differentiation. Usually ES cells are split at 1:5 to 1:7 dilution depending on their growth rate, ideally every other day (about  $1 \times 10^6$  cells are seeded onto 60-mm plate,  $2 \times 10^6$  cells onto 100-mm plate). Cells should be trypsinized to a single-cell suspension as large clumps might differentiate. The passage number of ES cells must be kept as low as possible with a stock of frozen vials kept in liquid nitrogen. It is recommended to create a frozen pool of low passage number of ES cells that serve as the only regular source of ES cells for many years of experiments.
5. For example, for 2 wells:  $\times 2.5$ , i.e., 2.5  $\mu\text{g}$  DNA in 600  $\mu\text{L}$  OPTI-MEM<sup>+</sup> 20  $\mu\text{L}$  LF in 600  $\mu\text{L}$  of OPTI-MEM.
6. Often cells in different wells might not grow at a synchronous rate. Some wells might not be subconfluent but have one or two colonies. Such wells can be trypsinized individually to allow more even growth after plating back the single-cell suspension. It is best to choose a time for the passage when the majority of the wells have reached 80% confluency. Ninety-six-well plates are split into 2 or 3 replica plates, which can then be passaged one more time if necessary.
7. For E12.5 and older embryos as well as tissues, the samples can be sectioned sagittally using a razor blade after 30 min of incubation at room temperature in freshly prepared prefix solution (2% PFA in PBS) and then fixed for an extra 30–60 min on ice.
8. If a plate has too many ES cell colonies that are also larger than necessary, they can be gently resuspended in trypsin. Aliquots of cell suspension can be transferred into a new plate with ES-DMEM after pipetting up and down 1–3 times to achieve clumps of the right size.
9. The goal is to create a small depression with a smooth surface, deep enough to hold the aggregate safely even when moving the plate to the incubator.
10. We usually aggregate 150–200 embryos (transferred into 8–10 recipients) per ES cell clone for germ line transmission. If chimeric embryos are dissected at mid-gestation to assess *in vivo* expression, two ES cell clones can be done in one experiment. As ES cells are derived from a pigmented mouse strain (in the case of R1 from an F1 hybrid of 129Sv-cp  $\times$  129 SvJ cross), and host embryos are derived from albino mice, the degree of chimerism can be estimated by eye pigmentation (from E11.5). Pigmented cells in the eyes are ES cell-derived and indicate overall ES cell contribution. By analyzing such chimeric embryos, a few candidate ES cell clones can be chosen for best contribution potential and gene expression for further aggregation for germ line transmission.

11. The AC field can be set up in advance; alternatively, slowly increase the AC field, so the embryos will orient properly.

## References

1. Nagy, A. (2000) Cre recombinase: the universal reagent for genome tailoring. *Genesis* **26**, 99–109.
2. Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E. J., and Manning, R. W. (1992) Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6232–6236.
3. Buchholz, F., Angrand, P. O., and Stewart, A. F. (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. *Nat. Biotechnol.* **16**, 657–662.
4. Rodriguez, C. I., Buchholz, F., Galloway, J., Sequerra, R., and Kasper, J. (2000) High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP [letter]. *Nat. Genet.* **25**, 139–140.
5. Groth, A. C., Olivares, E. C., Thyagarajan, B., and Calos, M. P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl. Acad. Sci. USA* **97**, 5995–6000.
6. Brocard, J., Feil, R., Chambon, P., and Metzger, D. (1998) A chimeric Cre recombinase inducible by synthetic, but not by natural ligands of the glucocorticoid receptor. *Nucleic Acids Res.* **26**, 4086–4090.
7. Kellendonk, C., Tronche, F., Casanova, E., Anlag, K., and Opherk, C. (1999) Inducible site-specific recombination in the brain. *J. Mol. Biol.* **285**, 175–182.
8. Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K., and McMahon, A. P. (1998) Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323–1326.
9. Gossen, M., Freundlieb, S., Bender, G., Muller, G., and Hillen, W. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**, 1766–1769.
10. Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., and Ullmer, C. (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA* **93**, 10933–10938.
11. Holzenberger, M., Zaoui, R., Leneuve, P., Hamard, G., and Le Bouc, Y. (2000) Ubiquitous postnatal LoxP recombination using a doxycycline auto-inducible Cre transgene (DAI-Cre). *Genesis* **26**, 157–159.
12. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat. Genet.* **21**, 70–71.
13. Lobe, C. G., Koop, K. E., Kreppner, W., Lomeli, H., and Gertsenstein, M. (1999) Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* **208**, 281–292.
14. Lewandoski, M. and Martin, G. R. (1997) Cre-mediated chromosome loss in mice. *Nat. Genet.* **17**, 223–225.
15. Friedrich, G. and Soriano, P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev.* **5**, 1513–1523.
16. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.
17. Joyner, A. (ed.) (1999) *Gene targeting: a practical approach*, 2nd ed., Oxford University Press, New York.
18. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993) Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428.
19. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the mouse embryo: a laboratory manual*, 2nd ed. series. CHS Laboratory Press, Cold Spring Harbor, N.Y.

20. Lawitts, J. A. and Biggers, J. D. (1992) Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol. Reprod. Dev.* **31**, 189-194.
21. Ho, Y., Wigglesworth, K., Eppig, J. J., and Schultz, R. M. (1995) Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol. Reprod. Dev.* **41**, 232-238.
22. Ramirez-Solis, R., Rivera-Perez, J., Wallace, J. D., Wims, M., and Zheng, H. (1992) Genomic DNA microextraction: a method to screen numerous samples. *Anal. Biochem.* **201**, 331-335.
23. Wood, S. A., Allen, N. D., Rossant, J., Auerbach, A., and Nagy, A. (1993) Non-injection methods for the production of embryonic stem cell-embryo chimaeras. *Nature* **365**, 87-89.
24. Kubiak, J. Z. and Tarkowski, A. K. (1985) Electrofusion of mouse blastomeres. *Exp. Cell Res.* **157**, 561-566.
25. Tarkowski, A. K., Witkowska, A., and Opas, J. (1977) Development of cytochalasin in B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *J. Embryol. Exp. Morphol.* **41**, 47-64.
26. Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., and Ivanyi, E. (1990) Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815-821.
27. Lobe, C. G. and Nagy, A. (1998) Conditional genome alteration in mice. *Bioessays* **20**, 200-208.
28. Mereau, A., Grey, L., Piquet-Pellorce, C., and Heath, J. K. (1993) Characterization of a binding protein for leukemia inhibitory factor localized in extracellular matrix. *J. Cell Biol.* **122**, 713-719.